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(54) Title: MAMMALIAN ARTIFICIAL CHROMOSOMES AND USES THEREOF

(57) Abstract

A circular nucleic acid vector – a mammalian artificial chromosome – which on introduction into mammalian cells replicates autonomously, is maintained extrachromosomally and is transmitted to daughter cells at cell division, the nucleic acid vector including a mammalian origin of replication and a mammalian centromere, and the nucleic acid vector not including a telomere functional in mammalian cells. The mammalian centromere may comprise alphoid DNA, especially including one or more CENP–B box sequences. A mammalian artificial chromosome may be produced by recombination between (i) a first vector including the mammalian origin of replication and mammalian centromere and (ii) a piece of DNA including the exogenous DNA sequence of interest, preferably within competent host cells.

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MAMMALIAN ARTIFICIAL CHROMOSOMES AND USES THEREOF

The present invention relates to mammalian artificial chromosomes (MACs), vectors which can replicate autonomously, are maintained extrachromosomally and are transmitted to daughter cells at cell division. Methods of construction and use of such vectors are provided in various aspects, especially wherein the vectors include an exogenous DNA sequence of interest, for example an expression cassette for expression of a desired product, such as a therapeutic protein.

Chromosomes are subcellular organelles including DNA and proteins which exist in a variety of forms during the cell cycle of nucleated cells. Although the bulk of the mass and the majority of the function resides in the protein components the purpose of the chromosome is to protect the DNA and to ensure its replication and delivery to daughter cells at cell division.

Despite the importance of the DNA component of chromosomes in terms of genes the critical elements required to distinguish a DNA molecule as a chromosome have remained largely undefined in higher eukaryotes until recently; a randomly chosen DNA molecule can be introduced into a mammalian cell but its fate is either loss from the cell or integration into an existing

chromosome.

This is not the situation in the yeast *S. cerevisiae* where a combination of biochemistry and genetics has been used to define replication origins, telomeres and centromeres as the minimal components of a chromosome.

Origins of replication are the sites at which bidirectional synthesis of daughter DNA strands initiates (Huberman, 1998). Telomeres are DNA sequences which promote stability of the ends of the chromosome (Cooke, 1996). A centromere is the site at which the complex machinery of the kinetochore assembles (Pluta et al., 1995) and which is responsible for segregation of the two copies of the chromosome to daughter cells.

All of these have been cloned, sequenced and are readily available as short, easily manipulable, DNA segments. When combined these have been used to construct vectors which act as Yeast Artificial Chromosomes, capable of carrying large fragments of exogenous DNA. This technology has been instrumental in generating large scale genetic maps and for introducing whole genes and control regions into transgenic mice. Such mice frequently show expression patterns from the gene contained in the now integrated YAC which mimic that of the endogenous gene.

These developments in yeast stimulated interest in defining equivalent components in mammalian cells. The first of these to be elucidated was the telomere which was rapidly shown to be functional when reintroduced into mammalian cells (Farr et al., 1991; Itzhaki et al., 1993) and showed many similarities with the telomere of yeast and other unicellular organisms (Blackburn, 1991). This similarity has facilitated a rapid expansion in understanding of the biology of telomeres which are now a focus of attention in the fields of cancer and senescence (Linskens et al., 1995).

Unfortunately the similarities seem to end there. Sequences that operate as origins of replication in mammalian systems have not been cloned as such although recent work is slowly clarifying the position (Aladjem et al., 1998). This has not however been a critical problem as, whilst there are between four and fifty centromeric sequences in a mammalian genome and twice that number of telomeric sequences, there are thought to be of the order of thirty thousand origins of replication. Effectively this means that any fragment of mammalian genomic DNA of at least about 100kb can be expected to to include an origin of replication.

Mammalian centromeres have been characterised in terms of their cytogenetics and biochemically in terms of some of their protein content.

Efforts to construct a mammalian chromosome have largely focused on the hypothesis that the repeated sequences found at most mammalian centromeres play a vital role, perhaps by forming a specific chromatin structure, and have used either heterochromatin or specific DNA sequences derived from heterochromatin as providing the basis of centromere formation.

Hadlzacky and co-workers (Praznovszky et al., 1991; Kereso et al., 1996) used an approach in which a mouse chromosome in which an amplification event which formed a long chromosome arm containing a variety of sequences including mouse major satellite and telomeric sequences was followed at some frequency by fragmentation of this amplified DNA giving rise to mini chromosomes. This approach is largely uncontrolled and depends on selection and screening to give mini chromosomes. The composition of these chromosomes is not well defined as their creation is dependent on a cascade of *in vivo* events.

Two more recent approaches have focused on use of alphoid DNA as centromeric sequences. Alphoid DNA's are present in a range of primates and have a basic structure consisting of a 171bp repeat (Wolfe et al., 1985). Variants occur within this repeat and within arrays of this repeat generating higher order patterns which make it possible to assign alphoid

sequences to species or to groups of chromosomes within a species or in some cases individual chromosomes. They are present at centromeres at the cytological level and for this reason alone have been considered prime candidates for centromeric DNA. An additional reason for their candidature is sequence conservation. In general, tandemly repeated sequences are not highly conserved between species, arguing against their having any biological function. However, one striking feature of much alphoid DNA is an eighteen base pair sequence which is recognised by a highly conserved protein CENPB (Pluta et al., 1995), a component of the human kinetochore. This sequence is also found in the mouse centromeric minor satellite and variants (Kipling et al., 1995) conforming to the minimal requirements for protein binding found in other species.

Initial attempts to transform alphoid DNAs into a variety of cell types gave equivocal results with some but not all properties of the centromere displayed by the alphoid DNA which integrated into a chromosome arm (Haaf et al., 1992). Many of these properties such as chromatid cohesion can be shown to be a feature of repetitiveness rather than being sequence dependent (Warburton and Cooke, 1997). These attempts did not involve the use of telomeres. The DNA was linearised.

Subsequently, with the development of more sophisticated cloning techniques in bacteria and yeast, different approaches have been applied all of which involve the use of telomeres.

Harrington et al. (Harrington et al., 1997) developed a method of constructing a unidirectional array of alphoid DNA starting from sequenced higher order repeats derived from either human chromosome 17 or Y. When mixed with telomeric sequences and total human DNA and transfected into a human cell line either as a simple mixture or after ligation, at least one mini chromosome was formed de-novo.

Ikeno et al. (Ikeno et al., 1998)used a different approach in which a 90kb alphoid array from a region of chromosome 21 rich in CENPB sites cloned as a Yeast Artificial Chromosome was fitted with mammalian telomeres by recombination and the gel purified chromosome introduced into a mammalian cell line giving rise to Mammalian Artificial Chromosomes.

The present invention is based on the surprising discovery that telomeres are not essential for generation of constructs containing centromeres (generally including alphoid DNA) which form stable episomes in mammalian cells.

Circular chromosomes (ring chromosomes) occur in human cells

in vitro and *in vivo*, but their DNA content is largely unknown, and circular episomes have been constructed based on Epstein Barr virus vectors (Sun et al., 1994; Vos et al., 1995). These EBV based molecules exist at high copy number per cell and segregate by virtue of association with metaphase chromosomes after breakdown of the nuclear envelope. They also are dependent on the presence in the cell of the viral gene product EBNA1. In contrast the episomes provided in accordance with the present invention may be limited in number to one or two per cell, need not contain any viral DNA and are not dependent on the expression of non host cell proteins.

As demonstrated by the experiments described below, other DNA, such as including expression constructs, may be introduced into the artificial chromosomes of the invention, for instance by simultaneously exposing host cells to the chromosomes and other DNA of interest in the form of a bacterial artificial chromosome (BAC) or phage P1 artificial chromosome (PAC). Co-transformation has been used in many contexts prior to this. A small proportion of a mammalian cell population in culture is capable of DNA uptake and stable incorporation, this proportion varying from cell type to cell type and with the method of DNA introduction. Those cells which are competent take up many molecules. Harrington et al. (Harrington et al., 1997) used this approach in the construction of their HAC's. It is surprising, however, that two circular DNA molecules

will co-integrate into each other and form a chromosome comprised of both components, as demonstrated herein.

The present invention generally provides various aspects relating to mammalian artificial chromosomes.

The present invention provides nucleic acid vectors which, in mammalian cells, replicate autonomously, are maintained extrachromosomally and are transmitted to daughter cells at cell division.

Vectors and artificial chromosomes of the invention include an origin of replication and a centromere, but do not include telomeres functional as such in mammalian cells. Vectors according to the invention are provided extracellularly and are introduced into cells as circular molecules, although they may be maintained within cells as circular or linear molecules.

A eukaryotic telomere consists of a DNA sequence repeat $(TTAGGG)^n$ where n is greater than 20 and generally at the end of a linear DNA molecule, or a variant sequence. The sequence is oriented such that the G rich strand runs 5' to 3' towards the end of the molecule.

By omitting telomeres as a component of the construction the

process is simplified and made more reliable. Furthermore, the enzyme which synthesises telomeres, telomerase, is not expressed in the majority of non-cancerous, non-transformed cell types. Previous telomere dependent mammalian artificial chromosomes may be found not to function in the vast majority of cells in normal animals and tissues. Artificial chromosomes according to the present invention may be stable in telomerase-negative and -positive cells.

The centromere DNA is generally alphoid DNA of a mammalian chromosome, for instance the α21-I region of human chromosome 21 (Ikeno et al., Human Mol Genet., 3, 1245-1257). The available mouse-human hybrid cell WAV17 (Raziuddin *et al.* (1984) *PNAS USA* **81**(17): 5504-5508) is a convenient source of human chromosome 21 DNA, although not essential for those skilled in the art.

Further convenient sources for human alphoid DNA are chromosomes 17 and Y, as used by Harrington et al. (Harrington et al., 1997), although in principle the alphoid DNA of any mammalian chromosome may be employed.

Experimental evidence is included below describing use of alphoid DNA from chromosome 21 and, separately, alphoid DNA of chromosome 17.

The nature of alphoid DNA (alpha satellite DNA) is reviewed in Willard and Waye, 1987, *TIG* 3(7): 192-197, which provides a consensus sequence based on comparison of 130 independent monomers derived from higher-order repeat units from at least 14 different human chromosomes. This paper notes an average 15-20% divergence of individual monomer sequences from the consensus, with the divergence being at particular positions in the repeat. Based on readily available information, including this paper, the ordinary person skilled in the art is unquestionably able to identify alphoid DNA of any chromosome, preferably human chromosome, of interest for inclusion as a functional centromere in a vector according to the present invention. The alphoid DNA employed in the present invention may include one or more (generally spaced repeats of) CENP-B box sequences studied by Ikeno et al, 1994, and for which a consensus sequence was estabished to be 5'-NTTCGNNNNANNCGGGN-3' (wherein N is any of A, T, C and G - Masumoto et al., 1993, NATO ASI Series, vol. H72, Springer-Verlag, 31-43; Yoda et al., 1996, *Mol. Cell. Biol.*, **16**: 5169-5177).

The present invention provides a circular nucleic acid vector including a mammalian origin of replication and a mammalian, preferably human, centromere comprising alphoid DNA. As noted the vector lacks a telomere.

The vector may include one or more sequences enabling its replication in yeast or preferably bacterial cells. Thus, for example, the mammalian origin of replication and centromere may be included in a bacterial vector. A P1 phage origin of replication and/or a yeast centromere may be included in a bacterial vector containing an alphoid DNA sequence.

The mammalian origin of replication and alphoid DNA may be contained within the same DNA sequence, i.e. the alphoid DNA may include an origin of replication. An origin of replication may be included within any convenient piece of mammalian DNA, such as within a coding sequence of interest included within the vector.

The use of bacterial vectors enables regulatory requirements for GMP DNA production to be met by established methods. In addition to quality, large DNA quantities are more easily obtained and the simple methods of manipulation involved increase the numbers of clones which can be obtained.

Vectors in accordance with embodiments of the invention may be introduced into mammalian cells to replicate autonomously, be maintained extrachromosomally and transmitted to daughter cells at cell division. That is to say the vectors may be used as mammalian artificial chromosomes (MAC's).

These chromosomes are stable, by which is meant that the chromosomes are not lost from all cells at cell division but segregate correctly in the absence of selection, so that they are maintained in the cell over at least about 30 days and/or about 25-30 generations in the absence of selection for the marker gene they carry, preferably at least about 60 days and/or about 50-60 generations, and more preferably at least about 90 days and/or about 80-90 cell divisions, such that after this number of generations at least about 33%, preferably about 50%, more preferably at least about 60%, more preferably at least about 70%, and more preferably at least about 75%, of the cells retain the chromosome, as detectable by *in situ* hybridisation and compared with the original cells following transformation.

Generally, the number of copies of the chromosome per cell is low, generally one, two or three, and may average one in a cell population. This is likely a reflection of the function of the constituent alphoid DNA in providing this aspect of centromere function. Copy number control has the advantage that the dose of a gene or genes provided by the chromosome is controlable. For many applications (see below) this will be important. In addition cells may only be able to support a limited number of centromeres without deleterious effects and so there are possible safety and efficiency implications.

An artifical chromosome according to the present invention may include an additional, exogenous DNA sequence of interest, for instance an expression cassette in which a sequence of interest is under the control of regulatory elements for expression

An expression cassette is a nucleic acid construct including nucleic acid with a sequence to be expressed, e.g. encoding a polypeptide of interest, and appropriate regulatory sequences for expression of the sequence in the relevant expression system, e.g. in eukaryotic cells such as COS or CHO cells, murine cells, human cells, cells of particular tissue type and so on.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et

al. eds., John Wiley & Sons, 1992.

DNA included in a vector of the present invention may include a coding sequence, e.g. cDNA, under the control of a heterologous promoter, i.e. a promoter not naturally associated with the coding sequence, or may include both structural and regulatory regions of a genomic DNA sequence.

An artificial chromosome including a mammalian origin of replication and a mammalian centromere and an additional sequence of interest, such as an expression cassette, may be produced by co-transforming competent host cells (which include HT1080 and HeLa cells) with an "empty" artificial chromosome and another piece of DNA which may be a "second" vector including the sequence of interest. Two vectors mixed outside the cell prior to DNA uptake by the cell are joined together in competent cells by a process which is not sequence dependent and is known generically as illegitimate recombination. This provides a facile method of introducing a gene or other DNA sequence of interest into the artificial chromosome without resort to complex biochemical manipulations such as restriction enzyme digestion and ligation or biological manipulation such as recombination in a yeast or bacterial cell.

A further aspect of the present invention therefore provides a

method of making a vector of the invention including a mammalian origin of replication and a mammalian centromere and an additional sequence of interest by means of recombination between a first vector including the mammalian origin of replication and mammalian centromere and another piece of DNA, which may be a genomic sequence or PCR product and may be a second vector including the additional sequence of interest. Such a method may include introduction of one of said first vector and further piece of DNA (e.g. second vector) into a competent host cell harbouring the other, or more preferably mixing of the pieces of DNA extra-cellularly before co-introduction (using any suitable technique) into a competent host cell wherein the recombination takes place. Following the recombination the vector including the origin of replication, centromere and additional sequence of interest (e.g. polypeptide encoding sequence) may be isolated and/or purified from the host cell. Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

Where the further piece of DNA is a second vector, it may be any suitable cloning vector such as a plasmid, bacteriophage, PAC, BAC or YAC, containing the DNA sequence of interest, such as a coding sequence for a desired protein or a transcribable sequence or provision of an RNA of interest (e.g. antisense

molecule or ribozyme). The second vector may also include regulatory sequences.

A further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein, preferably a mammalian cell and most preferably a human cell. Suitable host cells may be grown either in a culture system or *in vivo*, and preferably form single cells allowing cloning of cell lines. They are capable of DNA uptake using a suitable method, as discussed. Suitability of a host cell may be determined by means of an assay employing a reporter gene such as green fluorescent protein driven by an appropriate promoter in a construct. For recombination of pieces of DNA such as first and second vectors as discussed above, cells should be proficient in illegitimate recombination. Such cells are widespread, and examples include HT1080: fibrosarcoma, human: CCL 121 HeLa: epitheloid carinoma, cervix, human: CCL2 jprt-HT1080: this is an hprt-variant of the parent HT1080 cell line: CCL 121, MCF7: human breast cancer, HTB-22 ECACC number EJ138: human bladder carcinoma 85061108 Raji cells ACC CCL86.

See e.g. Schreiber Agus *et al.* (1997) *Current Topic in Microbiology and Immunology* Vol. 224; Wechsler *et al.* (1997), *Cancer Research* Vol. 57, No. 21, pp. 4905-4912; Fischer *et al.* (1998) *Cell Growth & Differentiation* Vol. 9, No. 3, pp.209-221; Fischer and Quinlan, *Journal of Virology* (1998) Vol. 72,

No. 4, pp. 2815-2824.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, microinjection, electroporation and liposome-mediated transfection. Lepofectamine (Gibco. BRL) may be used for transfections into mammalian cells in accordance with the manufacturer's instructions. Electroporation is discussed in the reference MacGregor, G.R., "Optimisation of electroporation using reporter genes" in *Guide to Electroporation and electrofusion* (1992) pp465-470, Academic Press Inc., New York, USA.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the product (e.g. polypeptide, antisense oligonucleotide or ribozyme) is produced. If a polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into surrounding medium.

Introduction of nucleic acid may take place *in vivo*, e.g. by way of gene therapy, as discussed below. A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place *in vivo* or *ex vivo*), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention.

Thus in various aspects the present invention further provides a non-human mammal including cells containing a construct as disclosed herein.

This may have a therapeutic aim. Gene therapy is discussed elsewhere herein. Furthermore, the presence in cells of an animal of a transgene within a construct according to the invention may allow the organism to be used as a model in testing and/or studying the role of the gene or substances which modulate activity of the encoded product *in vitro* or are otherwise indicated to be of therapeutic potential.

Vectors according to the present invention are useful in a number of practical applications. The capability of stable maintenance of a large DNA fragment regulatory elements for expression finds particular utility in gene therapy, biotechnology and animal genetics. Examples include large genes such as dystrophin, utrophin, clotting factor IX, Factor VIII, CFTR which may be provided for expression in a way controlled by the cell or organ, particularly if the complete gene (in excess of 100kb of DNA) and associated controlling regions are present in the vector. Other expression products of interest include cytokines, homeobox gene clusters with potential use in organ regeneration, tumour suppressor genes or apoptosis promoting genes for cancer therapy, complete globin gene clusters for globin-opathies. Similar sorts of genes may be expressed in cell culture for the production of the proteins for biotechnological or therapeutic application. The vectors may be used in transfer of histocompatibility genes to animals for humanising xenografts.

Other than including an open reading frame for expression of an encoded polypeptide, vectors according to the present invention may include sequences providing anti-sense transcripts or ribozymes for control of expression of one or more genes within a host cell. For instance an antisense nucleic acid molecule or ribozyme may be employed to reduce production of a mutant gene product.

Anti-sense oligonucleotides may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA or mature mRNA, interfering with the production of polypeptide encoded by a given DNA sequence (e.g. either native polypeptide or a mutant form thereof), so that its expression is reduce or prevented altogether. Anti-sense techniques may be used to target a coding sequence, a control sequence of a gene, e.g. in the 5' flanking sequence, whereby the antisense oligonucleotides can interfere with control sequences. Anti-sense oligonucleotides may be DNA or RNA. The construction of antisense sequences and their use is described in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990), and Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992).

Ribozymes are nucleic acid molecules, actually RNA, which specifically cleave single-stranded RNA, such as mRNA, at defined sequences, and their specificity can be engineered. Hammerhead ribozymes may be preferred because they recognise base sequences of about 11-18 bases in length, and so have greater specificity than ribozymes of the Tetrahymena type which recognise sequences of about 4 bases in length, though the latter type of ribozymes are useful in certain circumstances. References on the use of ribozymes include Marschall, et al. Cellular and Molecular Neurobiology, 1994. 14(5): 523; Hasselhoff, Nature 334: 585 (1988) and Cech, J. Amer. Med. Assn., 260: 3030 (1988).

Variation of expression of sequences due to integration position effects may be reduced by use of MACs to carry the sequences. Furthermore, problems of mutagenesis associated with unpredicatable insertion into nuclear chromosomes may be reduced by use of the vectors.

In further aspects the present invention relates to pharmaceutical compositions and uses involving constructs and host cells of the invention.

Thus, the present invention extends in various aspects not only to a nucleic acid vector or mammalian artificial chromosome as disclosed, but also a pharmaceutical composition, medicament, drug or other composition comprising such a vector or a host cell containing such a vector, a method comprising administration of such a composition to a patient, e.g. for delivery of a therapeutic polypeptide in treatment (which may include preventative treatment) of a disease, use of such a substance in manufacture of a composition for administration, e.g. for increasing delivery of a therapeutic polypeptide for instance in treatment of a disease, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Administration of a composition according to the present invention is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would

otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Aspects and embodiments of the present invention will now be illustrated further with reference to the following drawings and experiments. Further aspects and embodiments will be apparent to those of ordinary skill in the art.

All documents cited herein are incorporated by reference.

EXPERIMENTAL

EXAMPLE 1 - GENERATION OF MAMMALIAN ARTIFICIAL CHROMOSOMES

Construction of an alphoid containing PAC.

The 70 kbp human chromosome 21 derived alphoid repeat insert in Yac 7c5 (Ikeno et al., 1998) was transferred to the P1 artificial chromosome vector pPAC4 (Genbank U75992) as follows:

A NotI digest of 5 ug of total yeast DNA in an agarose plug was run on a CHEF pulsed-field LMP gel (low melting point, Gibco BRL) in sterile 0.5xTAE to separate DNA smaller than 50 kb from the remainder of the Not I fragments. A gel slice was removed which contained Not I cut total yeast DNA $\geq$ 50 kb

including the 7c5 insert. The gel slice was washed three x 20 minutes in agarase buffer (25mM Tris-Acetate pH 6.0, 100mM NaCl and 10mM EDTA) then melted at 68°C for 10 minutes. After mixing thoroughly but gently with a pipette tip an aliquot of 100 ul was removed with a cut-off pipette tip and agarased with 1.25 unit of agarase (Boehringer Mannheim) at 45°C for one hour. If properly agarased, the molten state should be retained after sitting on ice for 10 minutes. The molten agarose/DNA was dialysed in a microconcentrator tube (Millipore Ultra-MC, 10,000 NMWL) against T10E1,75 mM NaCl pH 8.0, for 30 minutes at room temperature, then transferred to a 1.5 ml tube and centrifuged under vacuum to a volume of 25 ul. Using the same microconcentrator, the solution was redialysed against 1 x ligation buffer (30mM NaCl, 50mm Tris-Cl pH 7.6, 10mM $MgCl_2$, 0.75mM spermidine, 0.3mM spermine) for one hour at 4°C. The DNA concentration was estimated at this time. This DNA was ligated to previously prepared Not I cut, LMP gel purified pPAC4 (no alkaline phosphatase treatment). Forty ng of yeast DNA was ligated to 30 ng of pPAC4 in a 25 ul reaction containing 1 x ligation buffer (as above), 1mM dithiothreiotol, 1mM rATP, and 400 units ligase (New England Biolabs) at 16°C for 16 hours. The ligation reaction was spot dialysed against $T_{10}E_1$ for 5 min. to remove salt before 2 ul was electroporated into 25 ul DH10B cells using standard conditions (BioRad Genepulser). The desired transformant (pPAC4/7c5) was obtained by replica plating colonies onto a

filter, releasing the DNA and screening with a probe specific to the alphoid sequence. An estimated 10^6 cfu/ug of electroporated DNA was obtained.

Generation of a mammalian artificial chromosome in HT1080 cells

pPAC4/7c5 DNA was obtained following culture of an inoculum in 100-200 ml of L-broth + 20 ug/ml kanamycin and purification on a Qiagen tip 100 according to the manufacturer's protocol for high molecular weight DNA. Typically 10 ug of DNA was obtained from 100 mls of broth under these conditions. One to two ug of DNA is recommended for lipofection, however the present inventors have used from 0.2 ug to 20 ug with similar results. Lipofection was performed according to the manufacturer's protocol (Gibco/BRL) to HT1080 cells (ATCC CCL121) at ~75% confluency in a T25 flask (~10^6 cells).

Following 8-12 hours in the lipofection media, the media was replaced with DMEM/FBS 10%. The next day the cells were transferred to one or two T80 flasks with fresh media. Blasticidine selection (ICN Biomedicals) was applied at 4 ug/ml of media (DMEM/FBS 10%) at 60-70 hours post lipofection (t_0= start of lipofection). Typically, several hundred blasticidine resistant colonies result, since pPAC4 carries the blasticidine-s-methylase gene. Colonies were picked to

establish cell lines, usually a dozen, and after expansion/storage, are prepared for scoring of artificial chromosomes.

Fixed chromosome preps for metaphase spreads were prepared for dual colour FISH as follows:

Seventy percent confluent cells in a T80 were exposed to media containing demecolcine (Sigma, Cat no D-1925, 100ul/10 ml of media) for 30-60 minutes in a tissue culture incubator. The cells were shaken off by rapping the flask against a surface several times and then collected to a tube and spun down. Resuspension was in 0.8mls of hypotonic 0.075M KCL and the cells were incubated 10-15 minutes at 37°C. The cells were washed 3 times in a 3:1 mix of MeOH:Glacial acetic acid, and stored at -20°C until ready for FISH.

FISH was performed according to standard protocols. Dual color FISH was employed to identify alphoid sequence and pPAC4 sequence on the candidate artificial chromosome. Chromosomes were counterstained with DAPI. The artificial chromosomes typically appeared 20%-25% the size of the smaller acrocentrics, but size varied.

The frequency of mammalian artificial chromosomes (MAC) varied between cell lines. Normally 30-40% of cell lines examined

were found to contain a MAC occurring in 30% to 100% of nuclei.

Characterisation of artificial chromosomes

pPAC/7c5 transfected as circular DNA generated artificial chromosomes of variable mitotic stability in HT1080 cells. These chromosomes are present in generally one or occasionally up to three copies per cell. Mitotic stability after 30 days of continuous passaging off selection occurs in some cell lines. Others show artificial chromosome loss which is significant at 30 days of passaging (see Table 1). In the example shown in Table 1, two cell lines show a fall of 25% from the original frequency of chromosomes after growth for 90 days. This corresponds to 80-90 cell divisions, in practice sufficient to produce a large mass of cells, indeed an animal. Inclusion of telomeres does not affect this outcome which is similar to that found using 7C5 alphoid DNA in a linear YAC vector as previously described (Ikeno, et al.). In one case where as a comparison telomere sequence was included in the alphoid PAC and transfected as a circle, mitotic stability extended to 90 days, but presence of telomere sequence did not guarantee increased stability (Table 2, T14c vs. T3.3c).

Frequency of detectable integration was low. Interstitial integration of PAC derived alphoid into host chromosomes was

never seen, however the pPAC4 vector could occasionally be found to integrate, more often in nuclei without artificial chromosomes. FISH with a telomere specific probe shows that the artificial chromosomes generated from pPAC4/7c5 (no telomere sequence added) have not acquired detectable telomere sequence from host chromosomes. Addition of telomere sequence to the PAC biased the integrations of PAC DNA towards telomeric ends. The size of the artificial chromosomes is variable but most commonly between 5-10 Mbp, or 50 to 100 times that of the input alphoid PAC DNA.

It is worth noting that should a cell line demonstrate a decline in chromosome content which is unacceptably great, cell lines containing stable versions of the chromosome may be derived by subcloning, passaging and selection by FISH analysis.

EXAMPLE 2 - ADDITION OF GENES BY CO-TRANSFECTION

20 ug of pPAC4/7c5 was mixed with 2 ug of a BAC containing a 170 kbp mouse genomic insert and co-lipofected to HT1080 cells. In two of six cells lines examined by dual FISH, there was evidence of incorporation of the mouse genomic DNA into ~90% of artificial chromosomes. Artificial chromosomes in each cell line were present in 80% of nuclei scored for both cell lines. One of these chromosomes has been very stable

after 30 days of passaging of selection (Table 2, Mqk3).

Thus, exogenous DNA sequences may be incorporated into the structure of the MAC by co-transfection. Generally, the alphoid containing vector (e.g. PAC or BAC) is mixed with the exogenous DNA-containing PAC at a molar ratio of 20:1 with at least 1 ug of the exogenous DNA-containing PAC being applied to the cells as described above for lipofection. Ratios below this are not excluded but may result in interruption of the alphoid array and integration into chromosomal arms. Both input molecules are circular which should help to minimise integration.

DNA from pPAC4/7c5 was mixed with DNA from a BAC containing the genomic sequences encompassing the hypoxanthine-guanine phosphoribosyl transferase gene (HPRT) and introduced into HT1080 cells deficient in HPRT. The cell line A6-p3-51 contained an artificial chromosome which contains HPRT genes detectable by *in situ* hybridisation. Using Northern blotting (Sambrook *et al.* (1989) Molecular Cloning. Laboratory Manuals, Chapter 7, Cold Spring Harbor Press) and probing for the presence of the HPRT messenger RNA, no HPRT mRNA was detected in the HPRT negative parental cell line, but the message was detected in the A6-p3-51 cells selected for the presence of the chromosome with blasticidine and was retained when the cell line was grown in the absence of selection for 60 days.

EXAMPLE 3 - GENERATION OF MAMMALIAN ARTIFICIAL CHROMOSOMES USING ALPHOID DNA OF HUMAN CHROMOSOME 17

The alphoid array contained within clone pPAC C2BS was employed. This alphoid array contains two higher order arrays, one of a 14mer of the basic 171 bp repeat unit and one of a 16mer, derived from human chromosome 17. The overall length of the array with the PAC was 150 kb.

This alphoid array was introduced into HT1080 mammalian cells and shown to form extrachromosomal artificial chromosomes in a significant number of the clones analysed.

TABLES

Table 1 shows results of a number of independent experiments in which the vector pPAC4 containing the alphoid DNA insert 7C5 was introduced into cells by lipofection. The proportion of cells retaining the minichromosome formed was determined by fluorescence *in situ* hybridisation (FISH) and determined after different time points in culture in the absence of selection for the selectable marker carried by the construct.

Table 2 shows results of analysis of independently derived cell lines produced by lipofection with pPAC47C5 simultaneously with a BAC containing a cloned insert of mouse

genomic DNA. The proportion of cells retaining the minichromosome was determined by FISH.

TABLE 1

Stability of artificial chromosomes from pPAC4/7c5

	series1	series2	series3	
cell line	10c	2.3c	3.3c	
	no tel	no tel	no tel	
Days				
0	33	80	78	% Mchr
30	30	70	68	
60	17	50	60	
90	11	46	52	

TABLE 2

Stability of artificial chromosomes with telomeric or genomic DNA

	series1	series2	series3	series4	
cell line	T14c	T3.3c	Mqk3	Mqk5	
	with tel	with tel	genomic	genomic	
Days					
0	36	65	80	80	% Mchr
30	33	53	78	63	
60	36	28			
90	36				

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Claims:

1. A circular nucleic acid vector which on introduction into mammalian cells replicates autonomously, is maintained extrachromosomally and is transmitted to daughter cells at cell division, the nucleic acid vector including a mammalian origin of replication and a mammalian centromere, and the nucleic acid vector not including a telomere functional in mammalian cells.

2. A nucleic acid vector according to claim 1 wherein the centromere comprises alphoid DNA.

3. A nucleic acid vector according to claim 2 wherein the alphoid DNA is human.

4. A nucleic acid vector according to claim 3 wherein the alphoid DNA is human chromosome 17 alphoid DNA.

5. A nucleic acid vector according to claim 3 wherein the alphoid DNA is human chromosome Y alphoid DNA.

6. A nucleic acid vector according to any one of claims 3 to 5 wherein the alphoid DNA includes one or more CENP-B box sequences.

7. A nucleic acid vector according to any one of claims 3 to

6 which includes one or more sequences enabling its replication in yeast or bacterial cells.

8. A nucleic acid vector according to any one of the preceding claims which on introduction into mammalian cells is maintained in the absence of selection over at least about 50-60 generations such that after this number of generations at least about 50% of the cells retain the vector.

9. A nucleic acid vector according to any one of claims 1 to 7 which on introduction into mammalian cells is maintained in the absence of selection over at least about 80-90 cell divisions such that after this number of generations at least about 60% of the cells retain the vector.

10. A nucleic acid vector according to any preceding claim including an exogenous DNA sequence of interest.

11. A nucleic acid vector according to claim 10 wherein the exogenous DNA sequence includes a coding sequence under control of a regulatory sequence for its expression.

12. A method of making a nucleic acid vector according to claim 10 or claim 11, the method including causing recombination between (i) a first vector including the mammalian origin of replication and mammalian centromere and

(ii) a piece of DNA including the exogenous DNA sequence of interest.

13. A method of making a vector according to claim 12 wherein the piece of DNA is a second vector.

14. A method of making a vector according to claim 12 or claim 13 wherein the method comprises mixing of the first vector and piece of DNA extra-cellularly before co-introduction into a competent host cell wherein the recombination takes place.

15. A method according to any one of claims 12 to 14 wherein said nucleic acid vector is isolated and/or purified from the host cell.

16. A method according to any one of claims 12 to 14 wherein the exogenous DNA sequence includes a coding sequence under control of a regulatory sequence for its expression and the method further comprises culturing the host cell for expression of the exogenous DNA sequence.

17. A method according to claim 16 further comprising isolating and/or purifying a product produced by expression of the exogenous DNA sequence.

18. A method according to claim 17 wherein the product is

formulated into a composition.

19. A method of producing a product, the method comprising culturing a host cell containing a nucleic acid vector according to claim 11 under conditions for expression from said exogenous DNA sequence.

20. A method according to claim 19 further comprising isolating and/or purifying a product produced by expression of the exogenous DNA sequence.

21. A method according to claim 20 wherein the product is formulated into a composition.

22. A mammalian host cell containing from one to three copies of a nucleic acid vector according to any one of claims 1 to 11.

23. A population of mammalian host cells according to claim 22 wherein the number of copies of the nucleic acid vector per cell in the population averages one.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 99/03227

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WOHLGEMUTH J G ET AL: "Long-term gene expression from autonomously replicating vectors in mammalian cells." GENE THERAPY, (1996 JUN) 3 (6) 503-12. , XP000857059	1-4, 7-11, 19-23
Y	the whole document	6
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
3 December 1999	16/12/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int: onal Application No
PCT/GB 99/03227

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Int tional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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